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Redundant and Alternative Roles for Activating Fc Receptors and Complement in an Antibody-Dependent Model of Autoimmune Vitiligo

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Summary

Complement and Fc receptor (FcR)-positive cells mediate effector functions of antibodies. Antibody-dependent immunity against the melanosome membrane glycoprotein gp75/tyrosinase-related protein-1 (TYRP-1) of melanocytes leads to autoimmune hypopigmentation (vitiligo) in mice. Hypopigmentation occurred in mice deficient in activating FcR containing the common γ subunit (FcγR $\gamma^{-/-}$) and in mice deficient in the C3 complement component. Mice doubly deficient in both FcγR γ and C3 did not develop hypopigmentation, suggesting that complement and FcγR formed redundant mechanisms. Following passive immunization with antibody, no further adaptive immune responses were required. Chimeric FcγR $\gamma^{-/-}$,$C3^{-/-}$ mice reconstituted with bone marrow from either FcγR $\gamma^{-/-}$ or $C3^{-/-}$ mice or adoptively transferred with FcγR $\gamma^{+/-}$ macrophages did develop antibody-mediated hypopigmentation. Thus, either complement or macrophages expressing activating FcγR can independently and alternatively mediate disease in a model of autoimmune vitiligo.

Introduction

Many autoimmune diseases are characterized by the presence of autoantibodies. The detection of autoantibodies can be required for a diagnosis of autoimmune diseases, and titer can reflect the severity of disease. Autoantibodies have been directly implicated in the pathogenesis of disease, e.g., autoimmune hemolytic anemia and thrombocytopenia, myasthenia gravis, and Grave's hyperthyroidism. Furthermore, experimental models of autoimmune disease have emphasized a crucial role for FcR in the pathogenesis of these inflammatory diseases (Ravetch and Clynes, 1998). Vitiligo is a prevalent and often disfiguring disease of acquired hypopigmentation that is due to destruction of cutaneous melanocytes (Nordlund and Ortonne, 1998). Melanocytes normally produce the pigment melanin in melanosomes, which are specialized vesicles found in the endocytic pathway (Vijayasaradhi et al., 1995). Melanosome membrane proteins of the tyrosinase family are cell type-specific molecules that play central roles in melanin synthesis, including determining the type of melanin synthesized. For instance, these glycoproteins are known to determine coat color in mice. A popular theory is that vitiligo is an autoimmune disease (Nordlund and Ortonne, 1998). In support of this notion, autoantibodies specific for melanosome membrane glycoproteins are found in sera of patients with vitiligo, including autoantibodies against tyrosinase (the *albino* locus product), TYRP-1 (*brown* locus), DOPAchrome tautomerase/TRP-2 (*slaty* locus), and gp100/Pmel17 (*silver* locus) (Song et al., 1994).

Passive or active immunization against the melanosome antigen TYRP-1 induces antibody-dependent hypopigmentation in mice (including C57BL/6, DBA/2, SJL, and C3H/HeN strains), showing that autoantibodies are sufficient to elicit vitiligo in otherwise healthy hosts (Hara et al., 1995; Clynes et al., 1998; Overwijk et al., 1999; A.N.H, unpublished data). In other experimental models of antibody-dependent autoimmune diseases, FcR have been directly implicated in disease pathogenesis. Activating FcR containing common γ chain, particularly the IgG low affinity activation receptor FcγRIII, is necessary in mouse models of autoimmune hemolytic anemia and thrombocytopenia, glomerulonephritis, collagen-induced arthritis, alveolitis, and Goodpasture's disease (discussed in Clynes and Ravetch, 1995). Specifically, mice deficient in FcγR γ chain are protected in these autoimmune models, and absence of inhibitory Fc receptors (FcγRIIB) can accelerate disease (Yuasa et al., 1999; Nakamura et al., 2000). However, in autoimmune models of vitiligo where C57BL/6 mice are actively or passively immunized against TYRP-1, preliminary studies have suggested that antibody-dependent autoimmunity after induction of either active or passive immunity surprisingly proceeds in the absence of FcγR γ chain (Clynes et al., 1998; Weber et al., 1998). Here we show that activating FcγR on macrophages is actually involved in the pathogenesis of experimental vitiligo but that the complement system provides an alternative pathway.

Results

Antibody-Induced Hypopigmentation

Antibody-dependent hypopigmentation in mice is induced after both active and passive immunization against the melanocyte glycoprotein TYRP-1 (Hara et al., 1995; Clynes et al., 1998, 2000; Weber et al., 1998;

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Table 1. Hypopigmentation in Mice Passively Immunized against TYRP-1

Mouse Strain	# Hypopigmented Mice/Total[a]
RAG-1$^{-/-}$	3/3
FcγR $\gamma^{-/-}$	15/15
CD4$^{-/-}$	5/5
Ig$\mu^{-/-}$	3/3
CR1/CR2	5/5
TcR$\delta^{-/-}$	3/3
FcγR $\gamma^{-/-}$, FcRII$^{-/-}$	3/3
FcRII$^{-/-}$	2/2

[a] Mice were depilated on day 0 and passively immunized with mouse mAb TA99 as described in Experimental Procedures. No vitiligo developed in control C57BL/6 mice passively immunized with isotype-matched mAb UPC10 (0/18 C57BL/6 mice).

Table 2. Hypopigmentation in Mice Passively Immunized against TYRP-1

Mouse Strain	Treatment	# Hypopigmented Mice/Total[a]
Wt C57BL/6	None	20/20
	UVB irradiated[b]	6/6
	anti-TNF-α mAb[c]	5/5
	UVB + anti-TNF-α	2/2
gld/*gld*C57BL/6	None	7/7
	anti-TNF-α	4/4

[a] Mice were passively immunized with mAb TA99 as described in Experimental Procedures.
[b] Mice were UVB irradiated with 2000 J/m on day 0.
[c] Mice were treated with anti-TNF-α mAb 2 hr before each TA99 injection.

Overwijk et al., 1999). In most mouse strains, including C57BL/6 mice, melanocytes are present in hair bulbs, and hypopigmentation manifests by profound dilution of coat color, particularly in regenerating hairs. The mAb TA99 is a mouse IgG2a autoantibody that reacts with TYRP-1 expressed by mouse melanocytes (Thomson et al., 1988; Vijayasaradhi et al., 1990; Hara et al., 1995). Antibody-dependent hypopigmentation was evaluated at depilated coat sites in regenerating hairs after passive immunization. In initial experiments, mice deficient in FcγR γ chain were able to develop hypopigmentation after passive immunization against TYRP-1 (Table 1), a phenomenon that we have also observed after active induction of autoimmunity against TYRP-1 in FcγR γ chain-deficient mice (Clynes et al., 1998; Weber et al., 1998). In addition, mice deficient in FcγRII and doubly deficient in both FcγR γ chain and FcγRII developed hypopigmentation. Hypopigmentation was not noticeably more severe in mice lacking inhibitory FcγRII receptors, and in all these deficient strains the severity of hypopigmentation was similar to that reported previously in wild-type C57BL/6 mice after either passive or active immunization against TYRP-1 (Table 1) (Hara et al., 1995; Clynes et al., 1998; Weber et al., 1998). Thus, activating FcR or combinations of activating and inhibitory FcR were not required for induction of disease.

Because autoimmune hypopigmentation can occur independently of FcγR, it was possible that antibody or antibody-antigen complexes might have recruited further adaptive immune responses downstream, e.g., elicitation of T cell responses through processing and presentation of antibody-antigen immune complexes or by induction of anti-idiotype responses. Experiments showed that no further involvement of adaptive immunity was necessary (Tables 1 and 2) because, first, neither T cells nor B cells were required for downstream mechanisms. RAG-1$^{-/-}$, CD4$^{-/-}$, TcR$\delta^{-/-}$ mice (expressed by intraepithelial dendritic T cells) (Tigelaar and Lewis, 1995), and Ig$\mu^{-/-}$ and CR1/CR2$^{-/-}$ mice (deficient in antibody responses to T cell-dependent antigens [Molina et al., 1996]) developed hypopigmentation (Table 1). Second, Langerhans cells in the epidermis were not implicated in uptake and presentation of antigen or antibody-antigen complexes because C57BL/6 mice exposed to ultraviolet B wavelength (UVB) radiation, which is known to impair cutaneous immunity by inducing death of epidermal Langerhans cells as well as intraepithelial dendritic T cells, did develop hypopigmentation (Table 2) (Tigelaar and Lewis, 1995).

It was possible that autoantibodies against TYRP-1 induced melanocyte death either directly or indirectly by triggering cell death pathways. We had previously shown that mAb TA99 does not induce any detectable target cell death or alteration in survival of cultured melanocytes (Takechi et al., 1996). Because transformed melanocytes can express fas (Hahne et al., 1996), a role for the fas-fas ligand (fasL) system in antibody-dependent immunity was examined. *gld*/*gld* mice developed hypopigmentation, ruling out a role for fasL (Table 2). In addition, systemic injection of neutralizing antibody against TNF-α, which is produced by keratinocytes and dermal infiltrating macrophages (Kock et al., 1990), did not block hypopigmentation even in *gld*/*gld* mice or in combination with UVB irradiation (Table 2). Normal cutaneous melanocytes are protected against programmed cell death in part through expression of bcl-2, so this result was not unexpected (Nakayama et al., 1994).

Antibody-Dependent Hypopigmentation Mediated through Either FcγR or Complement

Because FcγR were not required for autoimmune hypopigmentation, an alternative simple explanation was that complement activation was necessary, although this would go against the large body of experimental evidence for a primary role of FcR in antibody-dependent autoimmune diseases. However, C3$^{-/-}$ mice still developed hypopigmentation after passive immunization (Table 3). Mice deficient for complement receptor 1 and 2 also developed hypopigmentation (Table 1). These results are consistent with a mechanism that is independent of complement and these complement receptors.

Another explanation was that hypopigmentation was mediated through two distinct and alternative pathways involving either activating FcγR or complement. Three different types of experiments supported this scenario. In the first experiments, double-deficient FcγR $\gamma^{-/-}$,C3$^{-/-}$ mice were passively immunized against TYRP-1. Hypopigmentation could not be induced in these mice (Table 3 and Figure 1). This finding suggested that autoimmune vitiligo was established either through FcγR positive cells or the complement system.

Second, when FcγR $\gamma^{-/-}$,C3$^{-/-}$ mice were injected

Table 3. Lack of Hypopigmentation in Mice Deficient in Both FcγR γ and C3 and Hypopigmentation following Bone Marrow Reconstitution

Donor	Recipient/Treated Group	# Hypopigmented Mice/Total
None	$C3^{-/-}$	3/3
None	FcγR $\gamma^{-/-}$, $C3^{-/-}$	0/8
None	FcγR $\gamma^{-/-}$ + TA99 + C′[a]	3/3
None	FcγR $\gamma^{-/-}$ + TA99[b]	0/3
FcγR $\gamma^{-/-}$,$C3^{-/-}$	FcγR $\gamma^{-/-}$,$C3^{-/-}$	0/3
FcγR $\gamma^{-/-}$	FcγR $\gamma^{-/-}$,$C3^{-/-}$	3/3
$C3^{-/-}$	FcγR $\gamma^{-/-}$,$C3^{-/-}$	3/3

Mice were passively immunized with mAb TA99 as described in Experimental Procedures.

[a] Mice were passively immunized with mAb TA99 subcutaneously with sera from healthy C57BL/6 mice as a source of complement.

[b] Mice were passively immunized with mAb TA99 subcutaneously, but mouse serum was heated to 56°C for 30 min to inactivate complement.

cutaneously and subcutaneously with mAb TA99 plus normal C57BL/6 mouse serum as a source of complement, local hypopigmentation at the injected site was observed, but not when the mouse serum was heated to 56°C for 30 min to inactivate complement (Table 3), suggesting that complement was sufficient to induce autoimmunity in the absence of endogenous complement and activating FcR. These results showed that FcγR $\gamma^{-/-}$,$C3^{-/-}$ mice were competent to develop hypopigmentation.

In a third set of experiments, either FcγR or complement was shown to provide alternative pathways by transplanting bone marrow from mice genetically deficient in either FcγR γ chain or C3 complement component into lethally irradiated FcγR $\gamma^{-/-}$,$C3^{-/-}$ recipients. Mice reconstituted with bone marrow from FcγR $\gamma^{-/-}$ donors developed detectable serum C3 levels: levels were 605 ± 136 μg/ml (mean ± range) compared to 872 ± 164 μg/ml in wild-type C57BL/6 mice. Levels in FcγR $\gamma^{-/-}$,$C3^{-/-}$ and $C3^{-/-}$ mice were <3 μg/ml. On the other hand, donor marrow from $C3^{-/-}$ mice led to reconstitution of FcγR γ chain expression by hematopoietic cells, determined on mast cells and NK cells. Wild-type mast cells and NK cells normally express FcεRI and FcγRIII, respectively. However, in FcγR $\gamma^{-/-}$ mice neither receptor is expressed on these cell types. Bone marrow chimerism was predominantly donor origin in FcR $\gamma^{-/-}$,$C3^{-/-}$ mice receiving $C3^{-/-}$ donor marrow. A mean of 80% (range 65%–96%) of bone marrow-derived mast cells obtained from mice reconstituted with $C3^{-/-}$ (FcR $\gamma^{+/+}$) bone marrow were positive for FcεRI, and no expression of FcεRI (<1%) was seen on mast cells obtained from recipients of either FcγR $\gamma^{-/-}$ or FcγR $\gamma^{-/-}$,$C3^{-/-}$ bone marrow. Similarly, peripheral blood NK1.1$^+$ cells and IL-2-stimulated NK1.1$^+$ splenocytes were positive for FcRIII expression isolated from chimeric mice reconstituted with $C3^{-/-}$ marrow (67% FcRIII$^+$ versus 71% FcRIII$^+$ from wild-type C57BL/6 mice) but not with FcγR $\gamma^{-/-}$ or FcγR $\gamma^{-/-}$,$C3^{-/-}$ bone marrow (<1.5% FcRIII$^+$). Thus, bone marrow reconstitution was able to generate either complement or FcγR γ-chain positive cells in recipient mice.

Mice that were reconstituted with either FcγR$^+$ cells (after transplant with $C3^{-/-}$ marrow) or with C3 (after receiving FcγR$^{-/-}$ marrow) developed hypopigmentation after passive immunization (Table 3 and Figure 1). However, mice receiving marrow from FcγR $\gamma^{-/-}$,$C3^{-/-}$ donors showed no signs of hypopigmentation (Table 3 and Figure 1). Thus, these independent lines of evidence establish that autoimmunity is mediated either through activating FcR or complement.

Macrophages Expressing FcγR Mediate Antibody-Dependent Vitiligo

To address which bone marrow-derived cell type mediates vitiligo in this model, histologic sections of skin were examined. Mononuclear cell infiltrates were substantially increased in the dermis around hair follicles in mice passively immunized against TYRP1 compared to mice receiving isotype-matched mAb (Figures 2A and

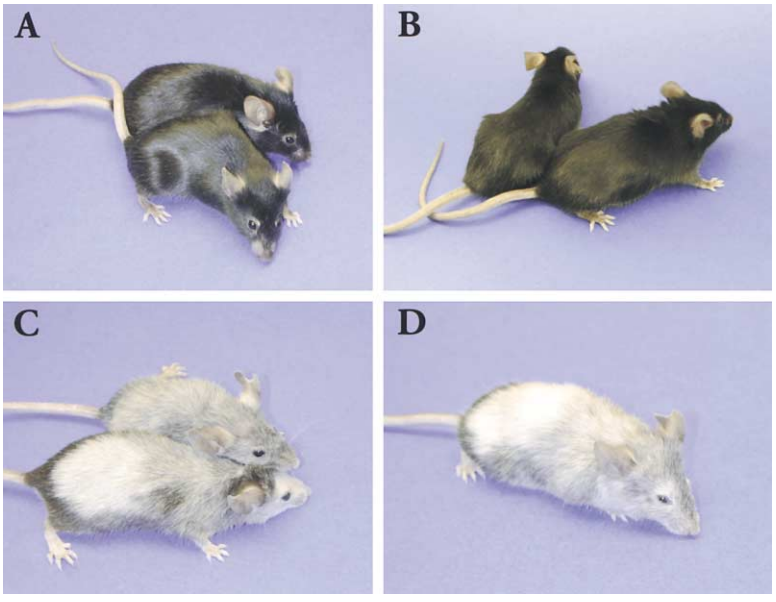

Figure 1. Hypopigmentation Not Present in FcγR $\gamma^{-/-}$,$C3^{-/-}$ Mice Passively Immunized with TA99 mAb against TYRP-1 and Rescued with Bone Marrow Reconstitution

(A) FcγR $\gamma^{-/-}$,$C3^{-/-}$ mice did not develop hypopigmentation.

(B) FcγR $\gamma^{-/-}$,$C3^{-/-}$ recipients transplanted with bone marrow from FcγR $\gamma^{-/-}$,$C3^{-/-}$ donors showed no hypopigmentation after immunization.

(C) FcγR $\gamma^{-/-}$,$C3^{-/-}$ recipients transplanted with bone marrow from $C3^{-/-}$ donors.

(D) FcγR $\gamma^{-/-}$,$C3^{-/-}$ recipients transplanted with bone marrow from FcγR $\gamma^{-/-}$ donors.

All mice were depilated over the posterior trunk where hypopigmentation is most prominent (see [C] and [D]), but when hypopigmentation occurred, it also spread outside the original depilated areas.

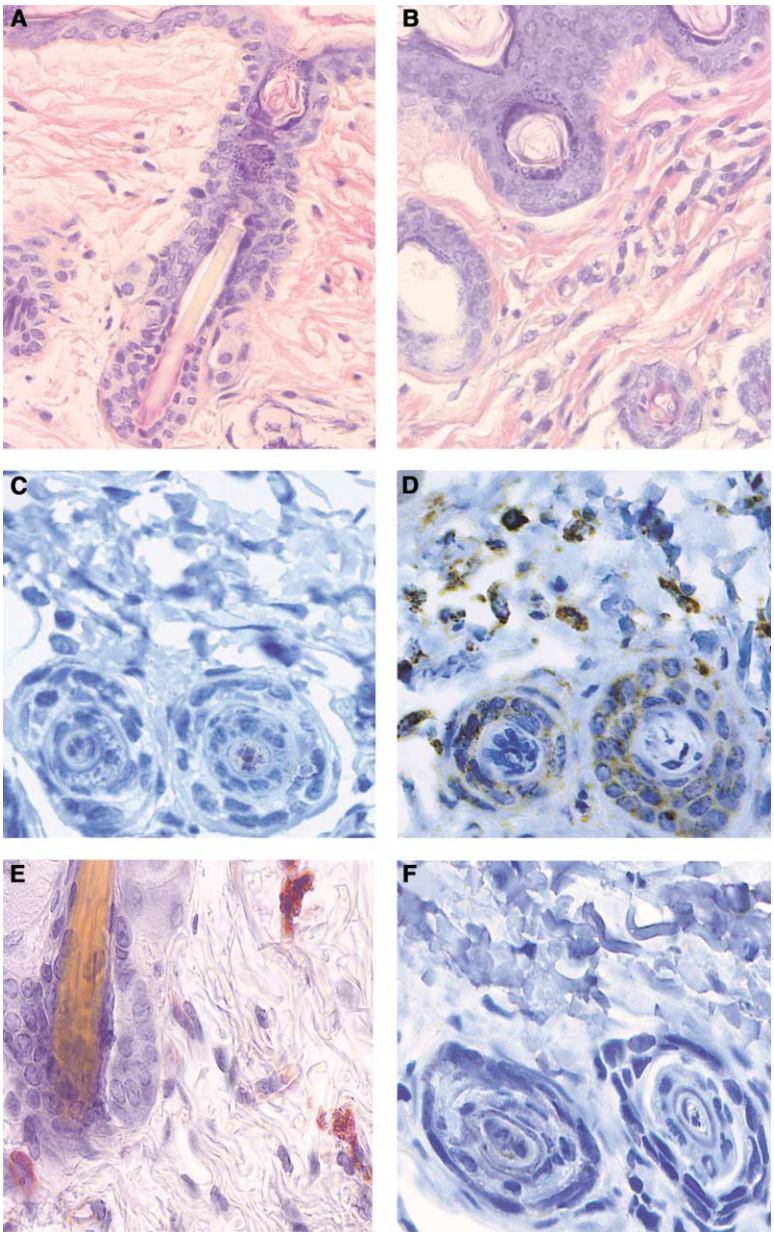

Figure 2. Histology of Epidermis and Dermis, Including Hair Follicles, of C57BL/6 Mice Passively Immunized with Either TA99 mAb against TYRP1 or Isotype-Matched mAb UPC10

Histologic sections are from mice passively immunized intraperitoneally with antibody every 2–3 days for five injections. Skin biopsies were obtained 3 days after the last antibody injection. (A) shows epidermis and dermis from mice receiving control mAb UPC10. Histology reveals minimal background mononuclear inflammatory cells in the dermis. (B), in contrast, shows a much more extensive mononuclear dermal infiltrate distributed about the hair follicle (where melanocytes are located in the mouse). (C) and (D) demonstrate the phenotype of inflammatory cells by immunostain with isotype-matched control mAb (C) or mAb against Mac-3 (D). Note the abundance of cells staining positively (brown) for Mac-3 distributed about the hair follicles. (E) shows staining of the cells for nonspecific esterase activity (magenta stain), confirming the macrophage identity of these mononuclear cells about the hair follicle. (F) shows that infiltrating cells do not express CD3. Magnification 40×. Sections are counterstained with hematoxylin.

2B). The mononuclear cell infiltrates were largely composed of cells with macrophage morphologies, interspersed with rare lymphoid cells. Phenotypic characterization showed that almost all infiltrating cells were Mac-3^+ by antibody staining and were positive for esterase activity (Leder, 1964) (Figures 2D and 2E) with the remaining rare infiltrating cells staining for $CD3^+$ (Figure 2F). Measurements of the inflammatory cytokines IL-1β, TNF-α, and MCP-1 in the skin of mice passively immunized with TA99 mAb were elevated but were not any different from control mice receiving isotype-matched control antibody (data not shown).

Adoptive transfer experiments were performed to confirm that macrophages expressing FcγR receptors were necessary for antibody-dependent vitiligo. To reconstitute the effector response, 2×10^5 FcγR $\gamma^{-/-}$ or $\gamma^{+/-}$ bone marrow-derived macrophages from $C3^{-/-}$ background mice were injected subcutaneously into vitiligo-resistant FcγR$\gamma^{-/-}$,$C3^{-/-}$ recipients in the presence of TA99 mAb or isotype-matched control mAb. Vitiligo was induced in an FcγR-dependent manner in TA99-treated mice. TA99-treated mice exhibited vitiligo after transfer of Fc$\gamma R^{+/-}$ macrophages, but no vitiligo was observed in mice receiving Fc$\gamma R^{-/-}$ macrophages (Table 4). The same incidence of vitiligo was observed in TA99-treated FcγR$\gamma^{+/-}$,$C3^{-/-}$ positive control mice without adoptive transfer of cells. Thus, FcγR-bearing macrophages were necessary to mediate vitiligo in vivo, showing that these cells can mediate autoimmune vitiligo in this model.

Discussion

A direct pathogenic role for immunity against melanosome glycoproteins has been shown in experimental mouse models of hypopigmentation (Hara et al., 1995;

Table 4. Requirement for FcγR γ^+ Macrophages in Vitiligo Follows Adoptive Transfer

Treated Mice	FcγR $\gamma^{+/-}$,C3$^{-/-}$ Mice	FcγR $\gamma^{-/-}$,C3$^{-/-}$ Mice	FcγR$^{-/-}$,C3$^{-/-}$ Recipients of Bone Marrow-Derived Macrophages	
Adoptive Transfer	No transfer	No transfer	FcγR $\gamma^{-/-}$ Macrophages	FcγR $\gamma^{+/-}$ Macrophages
TA99 mAb[a]	2/3	0/3	0/3	2/3
Control mAb[a]	0/3	0/3	0/3	0/3

[a] Groups of mice were passively immunized with TA99 mAb against TYRP-1 or isotype-matched IgG2a mouse mAb subcutaneously with or without adoptive transfer of macrophages as described in Experimental Procedures. Number of mice with hypopigmentation/total # mice treated.

Naftzger et al., 1996; Weber et al., 1998; Clynes et al., 1998; Bowne et al., 1999; Overwijk et al., 1999). The mouse models develop hypopigmentation of coat color, histologically characterized by loss of melanocytes in hair follicles and sparse leukocyte infiltrates (Hara et al., 1995; Weber et al., 1998; Overwijk et al., 1999). Cutaneous melanocytes in most mouse strains are restricted to hair follicles. However, in studies of transgenic mice that have epidermal melanocytes (which more directly reflects the physiology of melanocytes in human skin) (Kunisada et al., 1998), autoimmune cutaneous hypopigmentation has both clinical and histologic characteristics that directly mimic vitiligo in humans (J.T., J. Longeley, E. Carter, and A.N.H., unpublished data).

We describe mechanisms underlying a tissue-specific autoimmunity mediated by antibodies. Our results demonstrate that antibody-mediated responses, specifically mediated by IgG2a antibodies, can proceed through pathways that use either FcγR or complement. FcγR-independent mechanisms must underlie vitiligo induced by both passive and active immunization (Weber et al., 1998). The FcγR-dependent mechanism of vitiligo involves tissue macrophages. Evidence for involvement of FcγR-positive macrophages is also compelling for immunity against melanoma, which is transformed melanocytes (Clynes et al., 2000). One possibility is that both FcγR and complement-mediated pathways recruit macrophages as final effector cells, perhaps through CR3 for complement.

It has been proposed that FcR and complement systems evolved independently for different purposes (Ravetch and Clynes, 1998). FcRs participate in initiating inflammatory autoimmunity, and complement is important for innate immune responses such as against bacterial pathogens and toxins. Our results showed that both pathways can independently mediate a cutaneous autoimmune reaction. Potentially additive and codominant roles for FcR and the C5a component of complement previously have been suggested for Arthus reactions triggered by immune complexes of foreign antigen (i.e., rabbit anti-ovalbumin/ovalbumin), depending on the tissue site of inflammation (Hazenbos et al., 1996; Kohl and Gessner, 1999; Baumann et al., 2000). Cutaneous Arthus reactions elicited by immune complexes with foreign antigen using rabbit antisera are largely dependent on FcγR (varying with the mouse strain and baseline levels of complement), but inflammation triggered by immune complexes in the lung and peritoneum can be dependent on both FcγR and complement (Hazenbos et al., 1996; Baumann et al., 2000; Sylvestre et al., 1996). In particular, the complement component C5a is implicated through binding to the C5a receptor (Hopken et al., 1997; Kohl and Gessner, 1999; Heller et al., 1999; Baumann et al., 2000). Early complement components, like C3, are presumably not generally involved in the Arthus reaction, either in skin, lung, kidney, or synovium. In contrast, late components, like C5a, appear to be involved in synovium and perhaps other sites. In any case, our results show that these divergent pathways, FcR and complement, can clearly play a redundant role in an autoimmune model.

An identical phenotype of hypopigmentation can be also induced through CD8$^+$ T cell-dependent pathways that require perforin but are independent of antibody (Bowne et al., 1999). This means that the pathogenesis of hypopigmentation can proceed through at least three pathways: (1) antibody-dependent mechanisms involving activating FcγR, (2) antibody-dependent pathways using complement, or (3) cytotoxic T cells (and antibody-independent events). The mechanism of vitiligo in these models is dependent on the antigen used to induce autoimmunity. Immunity against TYRP-1 is mediated by autoantibodies, but immunity against the closely related paralogue TRP-2 is dependent on cytotoxic T cells (Weber et al., 1998; Bowne et al., 1999). These two antigens are closely related at the amino acid sequence level, and their expression is restricted to the same subcellular site in melanocytes. Thus, the immunogenic properties of closely related molecules can trigger quite distinct pathways leading to the same type of autoimmune disease. The major difference between these two molecules is small variations in amino acid sequence throughout the molecules. These small variations must be sufficient to initiate qualitatively very different mechanisms of autoimmune vitiligo. These distinct functional pathways can also form redundant mechanisms for autoimmune vitiligo in the case of antibody-dependent mechanisms. Blockade of both activating FcγR and complement (which proceed independently of cytotoxic T cells) or cytotoxic T cell responses (which proceed independently of antibody) will be required to overcome experimental autoimmune vitiligo.

Experimental Procedures

Mice and Cells

C57BL/6, C57BL/6*gld*/*gld*, CD4$^{-/-}$ (Rahemtulla et al., 1991), TcR$\delta^{-/-}$ (Itohara et al., 1993), Ig$\mu^{-/-}$ (Kitamura et al., 1991), and RAG-1$^{-/-}$ (Mombaerts et al., 1992) mice were obtained from Jackson Laboratory (Bar Harbor, ME); these genetically deficient mice were all on a C57BL/6 background. Black coat-colored complement receptor CR1/CR2$^{-/-}$ (Molina et al., 1996) and C3$^{-/-}$ mice (Wessels et al., 1995) were from a C57BL/6 x C57BL/6/129 background. FcγR γ^--deficient (Takai et al., 1994) and FcRII-deficient (Takai et al., 1996) mice have been described and were housed at Taconic Farms (Germantown, NY). FcγR γ-deficient and FcRII-deficient mice had been

crossed for twelve generations and nine generations, respectively, to a C57BL/6 background. Double-deficient FcγR $\gamma^{-/-}$,C3$^{-/-}$ mice resulted from the cross of these twelfth generation FcγR $\gamma^{-/-}$ backcrossed mice with C3$^{-/-}$ mice from a C57BL/6 x C57BL/6/129 background. Double-deficient FcγR $\gamma^{-/-}$, FcRII$^{-/-}$ mice were generated by crossing fourth generation backcrossed FcRII$^{-/-}$ and eighth generation backcrossed FcγR $\gamma^{-/-}$ C57BL/6 mice. Six- to eight-week-old mice were used for experiments.

Induction of Hypopigmentation with TA99 mAb

TA99 is a mouse IgG2a mAb specific for human and mouse TYRP-1 (Vijayasaradhi et al., 1990; Hara et al., 1995). TA99 was purified by protein G affinity chromatography (Pharmacia, Uppsala, Sweden). Mice were mechanically depilated over an ~1 cm^2 area at the center of the posterior flank under methoxyflurane anesthesia on day 0 and received intraperitoneal injections with 0.3 mg of TA99 or of the isotype-matched control mAb UPC10 (Sigma) on days 0, 2, 4, 7, 9, and 11. This dose of TA99 is 5-fold higher than the minimal dose required for inducing hypopigmentation (Hara et al., 1995); this dose was selected because it reproducibly induces prominent hypopigmentation (>50 of 50 C57BL/6 mice treated). Hypopigmentation was scored when >80% of regrowing hairs in depilated areas were white, but in control groups regrowth of depigmented hairs was always <1%. Irradiation with bone marrow transplantation could induce scattered white hairs in a salt and pepper pattern but never was >5% of hairs. In some experiments, mice were subcutaneously/cutaneously injected once with 0.3 mg of TA99 or UPC10, with or without 0.1–0.3 ml of normal mouse serum, at a depilated site. Injection of up to 1 mg of mAb UPC10 by either intraperitoneal or subcutaneous routes did not induce specific hypopigmentation.

Immune Manipulation of Mice

For ultraviolet B (UVB) irradiation, mice were depilated over an ~1 cm^2 area at the center of flank, then irradiated with an UV irradiator (Stratagene, La Jolla, CA) at a peak wavelength of 312 nm at 2000 J/m^2 on day 0. This dose of UVB irradiation induces disappearance of epidermal Langerhans cells at irradiated sites for 14 days (Odling et al., 1987). For neutralization of TNF-α, mice were injected intraperitoneally with 250 μg of neutralizing anti-TNF-α mAb 2E2 (Hernandez-Caselles and Stutman, 1993) 2 hr before each TA99 mAb injection.

Bone Marrow Chimera

FcγR $\gamma^{-/-}$,C3$^{-/-}$ mice received whole body γ irradiation of 100 cGy. Six to nine hours later, irradiated mice were injected intravenously with 5 × 10^6 bone marrow cells from FcγR $\gamma^{-/-}$,C3$^{-/-}$, FcγR $\gamma^{-/-}$, or C3$^{-/-}$ mice. Before injection, transplanted bone marrow cells were depleted of CD4$^+$ and CD8$^+$ cells with anti-CD4 mAb GK1.5 from the American Type Culture Collection (ATCC, Gaithersburg, MD) and anti-CD8 mAb, 2.43 (ATCC) in the presence of rabbit complement. Six weeks after bone marrow transplantation, TA99 treatment was started, concomitant with depilation. Serum C3 complement levels were measured 18 weeks after transplant by ELISA as described (Fischer et al., 1996).

Expression of FcR in bone marrow chimeras was assessed in both primary mast cells and NK cells. For mast cell isolation, bone marrow cells were cultured at 5 × 10^5 cells in 10% conditioned media of WEHI-3 cells (obtained from the ATCC, Gaithersburg, MD) as a source of IL-3. After 6 weeks, all mast cell cultures were uniformly positive for the mast cell marker c-kit by FACS analysis (Pharmingen clone ACK45; BD Pharmingen, La Jolla, CA). High affinity FcεRI expression on mast cells was assessed by staining with mouse IgE followed by a secondary FITC-anti-mouse IgE (Pharmingen clone R35-72). NK cells, which normally express only FcRIII but not FcRI or FcRII, were obtained from both peripheral blood cells and 10 day cultures of IL-2-stimulated splenocytes (obtained as described in Takai et al., 1994)). In brief, nylon-wool nonadherent splenocytes were cultured in 250 U/ml mIL-2 and 1 μg/ml indomethacin (Sigma, St. Louis, MO). Adherent cells from 10 day cultures or peripheral blood cells were assessed by flow cytometry after staining with phycoerythrin-labeled anti-NK antibody DX5 mAb against NK1.1 and the FcRII/III-specific antibody 2.4G2 labeled with fluorescein (both from Pharmingen). Chimerism was evaluated as the percentage of NK1.1 cells which were also positive for FcRIII. Chimerism was confirmed by the percentage of c-kit positive mast cells which were also positive for FcεRI by IgE staining.

Macrophage Adoptive Transfer Experiments

Bone marrow-derived macrophages were obtained by growth of nucleated bone marrow cells in 10% L929 conditioned media. In brief, bone marrows were flushed with a 25 gauge needle in RPMI medium, RBCs were lysed, and cells were cultured at 5 × 10^5/ml in DMEM/glutamine/10% FBS and 10% L929 conditioned media. Adherent cells were harvested at day 6 of culture with ice-cold PBS/5mM EDTA. These cells were uniformly >99% positive for expression of Mac-1 by flow cytometry (clone M1/70, Pharmingen). After extensive washing in PBS, macrophages were resuspended at 10^6 cells/ml. Mice were depilated immediately prior to intradermal injection of 200 μg TA99 and were subsequently injected subcutaneously with 200 μl of macrophages (2 × 10^5 cells/mouse). Evidence of depigmentation was observed as early as 3–4 weeks later upon hair regrowth but was definitively scored at 10 weeks after injection.

Genotype Analysis

Genotypes of all transplanted mice were confirmed by PCR analysis of tail-tip DNA both before and after transplant using FcγR γ primers and conditions as described (Clynes et al., 1998). For C3 PCR analysis, the following primers were used: C3 5′(5′-CTTCATAGACTGC TGCAACCA-3′), C3 3′(5′-AACCAGCTCTGTGGGAAGTG-3′), and neo-5′ (AAGCCGGTCTTGTCGATCAG). Annealing temperature was 56°C. Expected products were 950 bp for the wild-type and 900 bp for the disrupted allele.

Histologic Analysis

For identification of cell infiltrates, purified rat anti-mouse Mac-3 (clone M3/84, BD Pharmingen) at a concentration of 1μg/ml served as primary antibody. As the isotype control for Mac-3, purified rat IgG1 immunoglobulin isotype control (R3-34, BD Pharmingen) was employed. Whole goat serum was used as the blocking serum, and biotinylated mouse anti-rat IgG1/2a antibody, multiple absorbed (BD Pharmingen), was used as secondary antibody. As an additional means of macrophage identification, Leder's esterase method was utilized (Leder, 1964). To identify T cells, rabbit anti-human T cell CD3 (Dako Corp., Carpinteria, CA) at a 5μg/ml concentration was used, and rabbit immunoglobulin fraction (X0903, Dako) was used as the isotype control. With this primary antibody, whole goat serum (ICN Biomedicals, Aurora, OH) was utilized as the blocking serum and biotinylated anti-rabbit IgG made in goat (Vector Laboratories, Burlingame, CA) at a 3 μg/ml concentration as the secondary antibody.

The avidin-biotin peroxidase method was used for immunohistochemistry. Sections cut at 4 μm were heated at 55°C overnight and then deparaffinized and quenched with 3% hydrogen peroxide to block endogenous peroxidase activity. Sections were immersed in 0.01 M citric acid buffer (pH 6) in a microwave oven for 5 min. Each section was incubated for 30 min with 10% goat serum. Primary antibodies were applied and incubated at 4°C overnight. After extensive washing, sections were incubated at room temperature with the appropriate secondary antibody for 30 min. The sections again were thoroughly washed and then incubated with avidin-biotin peroxidase complexes (Vector Laboratories). Diaminobenzidine liquid chromogen 0.1% (Dako) was used as the final chromogen. Slides were counterstained with hematoxylin. For measurement of inflammatory cytokines in skin, skin biopsies were snap frozen in liquid nitrogen and homogenized. ELISAs for IL-1β, TNF-α, and MCP-1 were performed according to the manufacturer's instructions (Endogen Inc., Woburn, MA).

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